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Satoru Take*

*Okayama University,

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Satoru Take

Abstract

1. Mitochondria isolated from human liver, hepatoma and gastric cancer contain DNA. The DNA content per mitochondrial protein is about ten times as much in cancer as in normal liver. 2. Human liver, hepatoma and gastric cancer contain circular DNA molecules in their mitochondria. Circular DNAs from normal liver and cancer mitochondria are mostly about 5 μ long, and the frequency of circular DNAs of multiple or shorter length is higher in cancer mitochondrial DNA. The outline of the present paper was presented at the 26th Congress of Japanese Cancer Association (1967) (52, 53).

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DNA'S FROM HUMAN HEPATOMA AND GASTRIC CANCER MITOCHONDRIA

Satoru TAKE

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. T. Oda)

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In the past few years, DNA-like fibers have been observed in mitochondria under the electron microscope (1), and DNA-polymerase activity (2, 3), RNA-polymerase activity (4—6), ribosomes (7) and t-RNA (8), which all seem to be specific in mitochondria, have been demonstrated. Protein precursors (4—6, 9) and nucleic acid precursors (10, 11) are shown to be actually taken into mitochondria. Thus mitochondrial protein synthesis and self-duplication have aroused interest of many workers (12—14).

Since LUCK demonstrated the presence of DNA in isolated mitochondria from *Neurospora crassa* (4), many data on various organisms have been presented, suggesting that mitochondria contain their specific DNA. According to these data, mitochondrial DNAs differ from nuclear DNA in physical, chemical and morphological properties (15—20, 28—34).

On the other hand, in the field of oncology, the phenotypic expression of cancer cells with regard to mitochondria has been widely investigated in relation to respiration, oxidative phosphorylation, glycolysis, fatty acid oxidation and morphology (21—27).

The author extracted mitochondrial DNAs from human liver, hepatoma and gastric cancer. By the electron microscopy of these DNAs, it is demonstrated that human liver mitochondria contain circular-form DNA molecules measuring about 5 μ in contour length which are quite similar to those reported in other organisms and that human hepatoma and gastric cancer mitochondria also contain circular-form molecules, the majority of which are similar to those from normal liver mitochondria. In addition, smaller and longer circular-form DNA molecules were observed.

In this report, some quantitative as well as electron microscopic data on mitochondrial DNAs are shown and discussed concerning the heterogeneity of cancer mitochondrial DNAs.

MATERIALS AND METHODS

Livers and hepatomas were sampled in three hours after death from autopsy

materials, which had never received anti-tumor agants or x-ray therapy. Gastric cancers were sampled from the materials immediately after operation. Diagnosis was made by pathologists on all samples microscopically.

Mitochondria were isolated from the materials, which were pretreated to eliminate necrotic tissue and connective tissue, by the method of HOGEBOOM and SCHNIDER (35) for normal livers. For cancer tissues, proteinase treatment (1 mg/g tissue wet weight, for 15 min.) was carried out immediately before homogenization, and then HOGEBOOM and SCHNEIDER's procedure was followed. These crude mitochondria fractions obtained in 0.25 M sucrose were layered on 0.34 M sucrose and centrifuged (700×g, for 10 min.) several times to avoid nuclear contaminations. Each step of fractionation was checked under a phase-contrast microscope for its contaminations, and the purity of final mitochondria fraction was examined with an electron microscope. Nuclei were isolated by the method of CHAUVEAU, MOULE and ROUILLER (36).

Total nucleic acids were extracted by the method of SCHMIDT and THANHAUSER (37). DNA was assayed by the method of BURTON (38), and RNA, by orcinol reaction (39), using calf thymus DNA (Sigma) and D-Ribose (KATAYAMA) as standards.

Protein was measured by LOWRY's method (40) using bovine serum albumin as a standard.

Native DNA for electron microscopy and absorption spectra was extracted by the method of MARMUR (41) with slight modifications as follows.

a) At the step of alcohol precipitation, since mitochondrial DNA did not spool on the glass rod, DNA-alcohol mixture was left in an ice box over night and DNA was recovered by centrifugation.

b) At the final step of iso-propanol precipitation, samples were too small to pass through the step and this step was omitted.

RNase T_1 was donated by Dr. T. ARIMA at Prof. P. EGAMI's Laboratory, Department of Biological Chemistry, School of Science, University of Tokyo, and RNase I was purchased from Sigma Pharma. Co. LTD.

Absorption spectra of DNA were recorded in SSC (0.15 M NaCl, 0.015 M citrate. pH 7.0) using a Hitachi EPS-3T automatic spectrophotometer.

Electron microscopic observations on DNA was carried out following KLEINSCHMIDT and FLEIFELDER's method (42). Rotatory shadow was cast with platinum-paradium (80 : 20, about 20 mg in weigth) at the angle of 6—10°. Hitachi HU-11C type electron microscope was used.

Contour length of DNA was measured on the prints which were magnified 4 times (final magnification, 40,000) with a map-measuring device (42).

RESULTS

DNA content of normal liver and cancer mitochondria

It was shown electron microscopically that mitochondria from normal liver were highly purified but mitochondria from cancer contained micro-

somal and nuclear contaminations to some extent. DNA content of cancer mitochondria was about 10 times as much as that of normal liver mitochondria (Table 1).

Table 1 DNA content per mg. of mitochondrial protein of human liver, human hepatoma and gastric cancer mitochondria

Sources	DNA content, μg/mg. prot.
Human liver	0.5
Human hepatoma	7.3
Human gastric cancer	5.1

Absorption spectra of normal liver and cancer mitochondrial DNA

DNA extraction as descibed in METHODS AND METERIALS contained about 3 % of RNA and about 1 % of protein.

Wave lengths of the maximal and minimal absorption of nuclear and mitochondrial DNAs were 259 mμ, 232 mμ and 257 mμ, 230 mμ respectively. Mitochondrial DNAs showed a slight shift to shorter were length

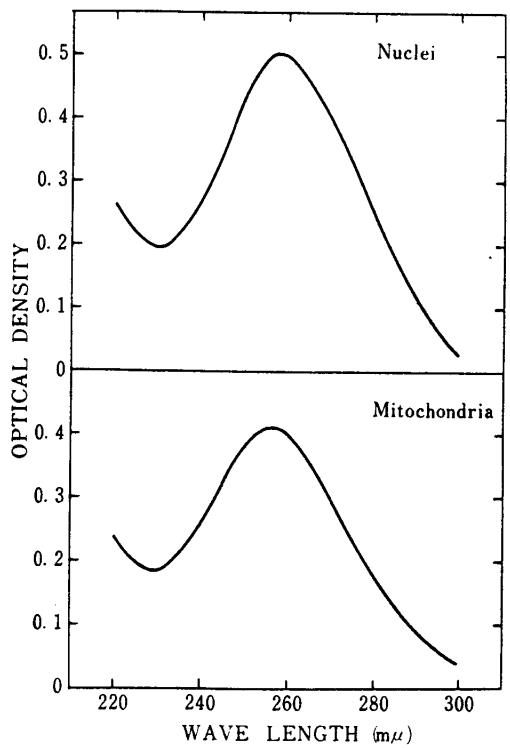


Fig. 1 Absorption spectra of nuclear and mitochondrial DNAs from human hepatoma

in their absorption peak. No difference was found between normal liver DNA and hepatoma or gastric cancer DNA (Fig. 1).

Electron microscopy of DNA molecules

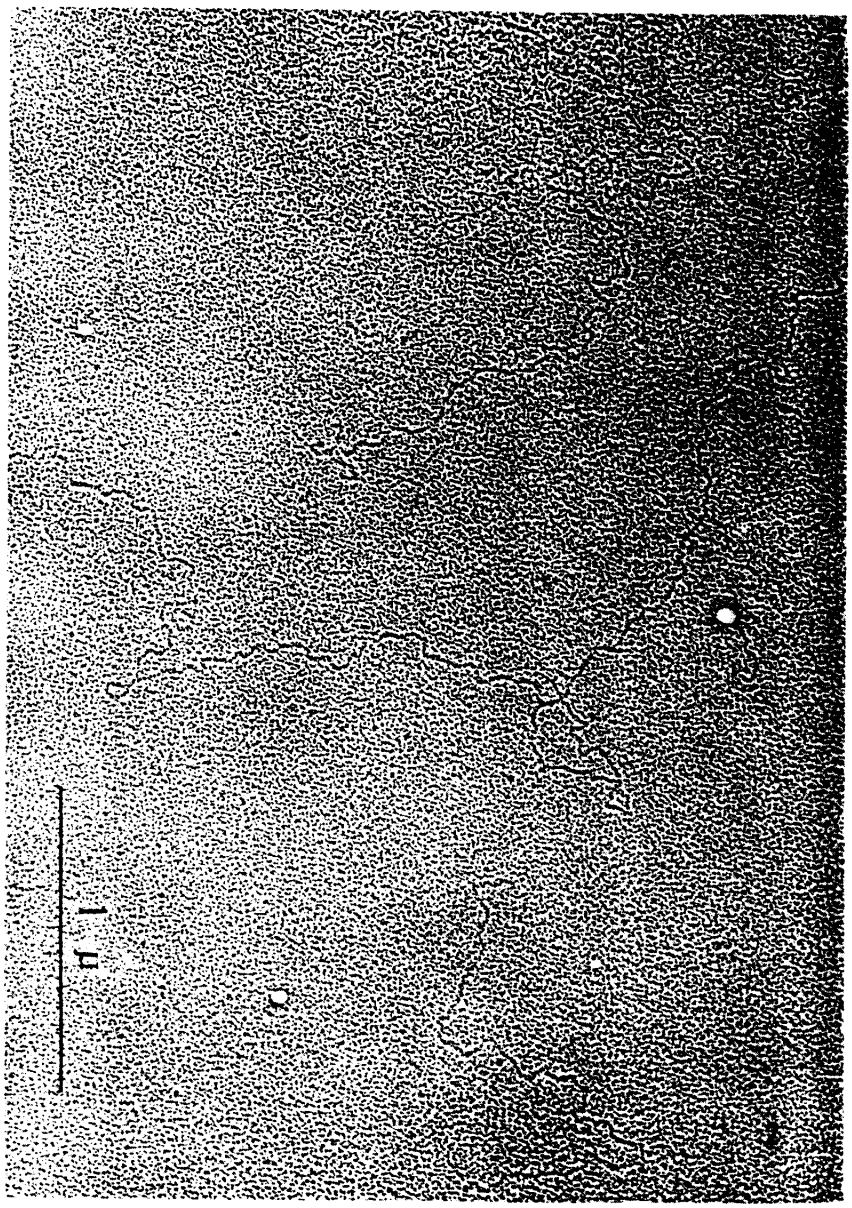


Fig. 2 Nuclear DNA from human hepatoma. Many linear DNA molecules of various length were observed. No apparent circular DNA molecule was discernible.

a) *Electron microscopy of DNA molecules from nuclear fraction*

Abundant linear DNA fibers of various length measuring from 0.5 μ to 40 μ were observed both on normal liver nuclear DNA and on hepa-

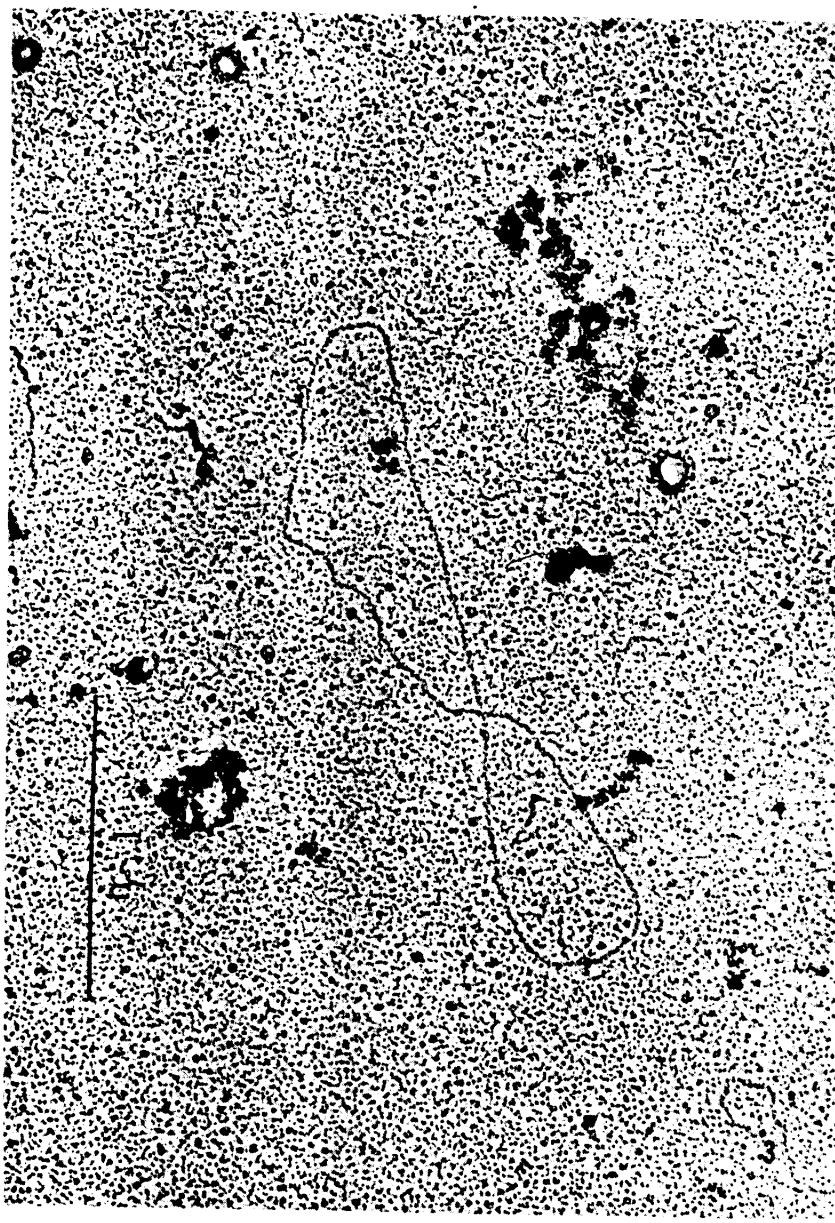


Fig. 3 Mitochondrial DNA from liver. A twisted circular DNA molecule is shown here.

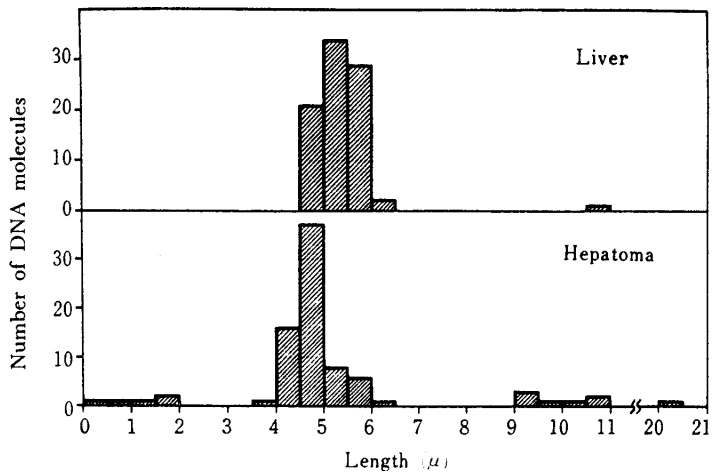


Fig. 4 Contour length distributions of circular DNA molecules from human liver mitochondria and human hepatoma mitochondria. Total number of DNA molecules: 87 in liver and 90 in hepatoma. Mean value of the highest frequency group: $5.32 \pm 0.4 \mu$ in liver and $4.81 \pm 0.46 \mu$ in hepatoma.

toma nuclear DNA. The length of DNA molecules seemed to be affected by the procedures of extraction and time until extraction after death. The width of DNA was about the same as reported on other double stranded DNA molecules. No apparent circular DNA molecules were discernible (Fig. 2).

b) *Electron microscopy of DNA molecules from normal liver mitochondria*

About half of DNA molecules showed circular configurations. Circular DNA molecules came in various shapes such as open circles, twisted circles and supercoiled circles (Fig. 3). Almost all the circular DNA molecules were $5.32 \pm 0.40 \mu$ in contour length, and only a few DNAs measured 8—10 μ (Fig. 4). Linear DNA molecules contained small numbers of molecules longer than 5 μ which seemed to be derived from nuclear DNA. Almost all the linear DNA molecules were within 5 μ in length, while the majority of them averaged less than 2 μ (Fig. 5).

c) *Electron microscopy of DNA molecules from hepatoma mitochondrial DNA*

The ratio of the circular to the linear DNA molecules varied from 1 to 0.5. Circular DNA molecules showed their main peak at $4.81 \pm 0.46 \mu$ in the histogram. Also about 10 % of all the circular DNA molecules showed multiple lengths of main peaks, such as $9.82 \pm 0.39 \mu$, 14.6 μ, 20.5 μ. Moreover, small numbers of shorter circular DNA molecules

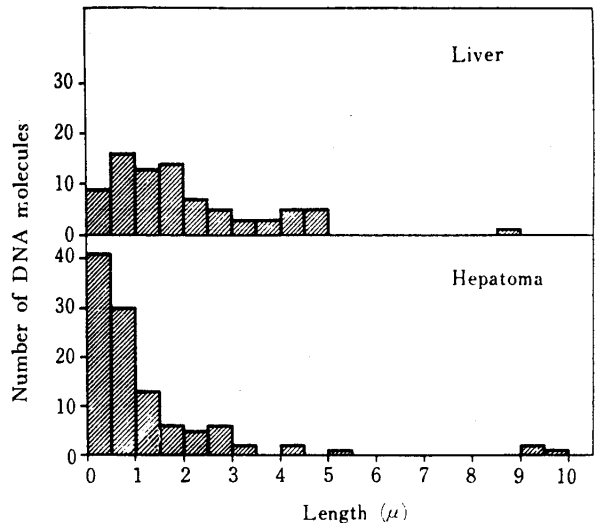


Fig. 5 Length distributions of linear DNA molecules from human liver mitochondria and human hepatoma mitochondria. Total number of DNA molecules: 81 in liver and 108 in hepatoma.

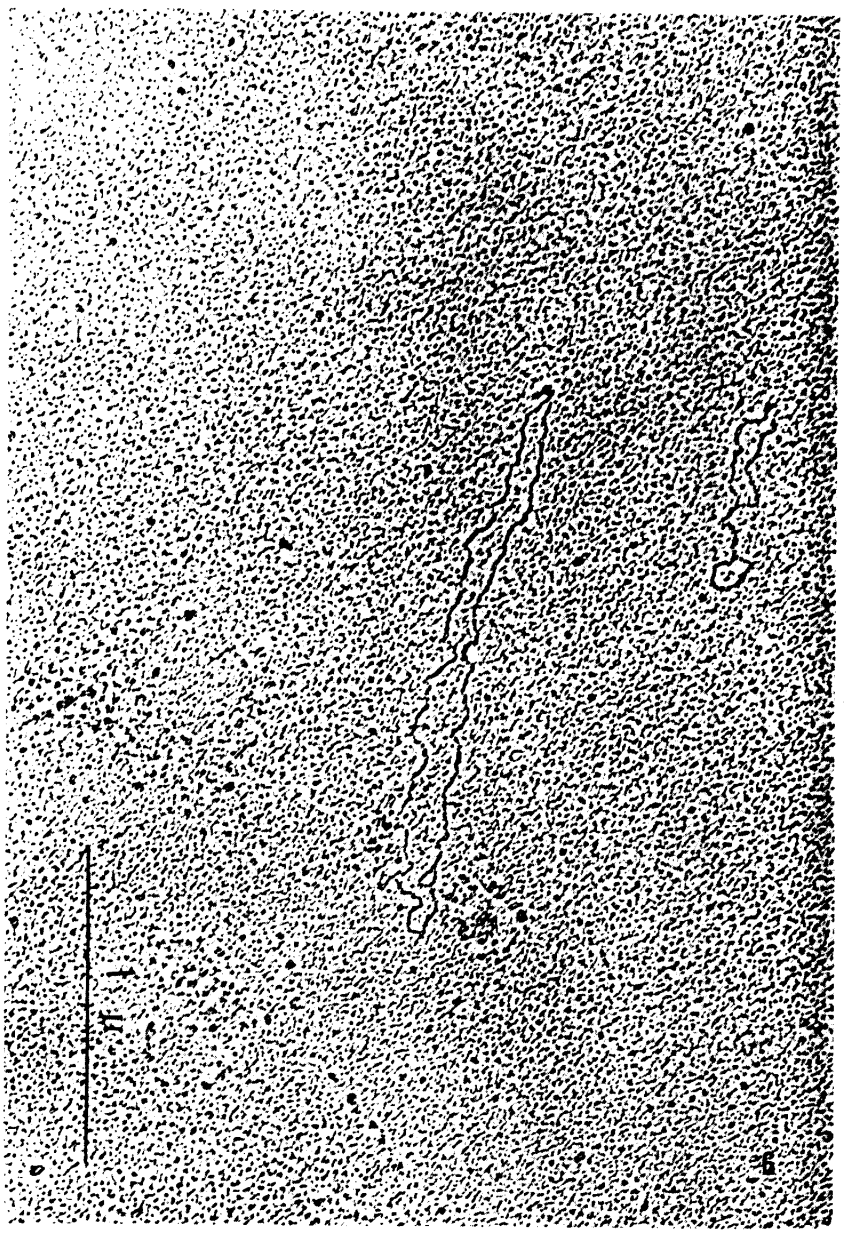
were observed, which ranged from 0.5 μ to 1.5 μ in contour length (Figs. 4, 6, 7). Circular DNAs of the main peak appeared in various shapes: open, twisted and supercoiled circles, and more twisted circles in comparison to normal liver mitochondrial DNA. Consequently the measuring of the contour length was more difficult. Circular DNAs of multiple lengths appeared only as twisted circles with smaller circles being open and twisted. The linear DNA molecules were mostly within 5 μ in contour length, predominantly around 1 μ.

d) *Electron microscopy of DNA molecules from gastric cancer mitochondria*

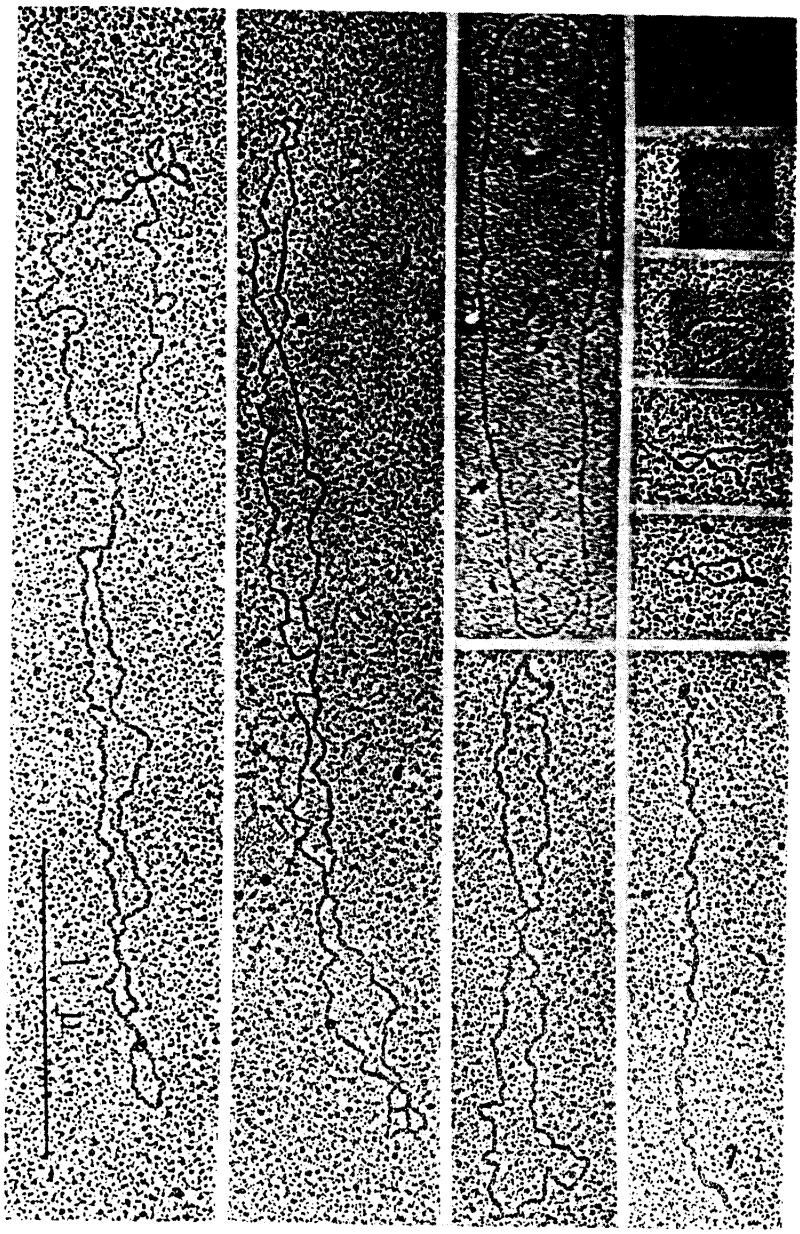
These samples showed many linear DNA molecules longer than 5 μ. It is most probable that these longer DNA molecules reflect the nuclear contaminations into mitochondrial fractions. But small numbers of circular DNA molecules have their main peak at 5.07 ± 0.19 μ (Fig. 8) and also longer molecules (8.65 μ) or smaller molecules (0.8, 1.3 μ) were observed.

DISCUSSION

It has already been reported that the HeLa cell, Chang cell and human foetus cell contain DNA-like fibers in their mitochondria according to electron microscopic investigation with sectioned tissue specimens (1). In the present report, it has been shown that mitochondria isolated from



Figs. 6, 7 Mitochondrial DNA from human hepatoma. Most of the circular DNA molecules are about 5 μ in length but small number of longer and shorter circular DNA molecules are discernible.



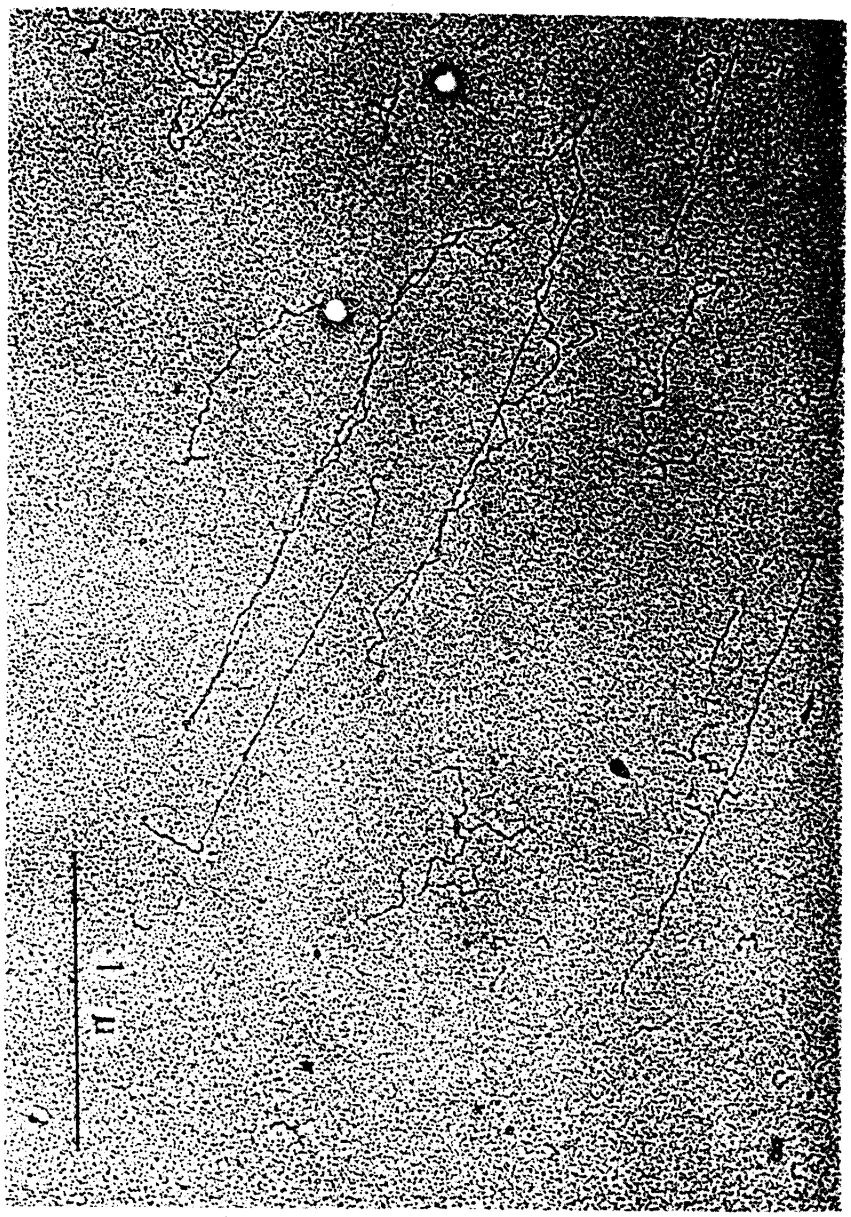


Fig. 8 Mitochondrial DNA from human gastric cancer. These DNA preparations contained many linear DNA molecules. Small number of circular DNA molecules are observed to show similar pattern of length distribution as that of hepatoma mitochondrial DNA.

human liver, hepatoma and gastric cancer contain a little but consistent quantity of DNA. Human liver mitochondrial DNA contents per protein agrees approximately with those reported on other organisms (17, 18, 20, 31). Cancer mitochondrial DNA content per protein is about 10 times as much as that of liver mitochondria. The problem whether DNA content per mitochondrion is actually increased in cancer remains to be decided since cancer cell mitochondrial fractions indicate some nuclear contaminations, and cancer cell mitochondria are generally smaller than normal cell mitochondria. In so far as the absorption spectra of DNAs are concerned, no difference between normal and cancer mitochondrial DNA was apparent. It has been demostrated by the electron microscopy that human liver, hepatoma and gastric cancer mitochondria contain circular DNA molecules, as VINOGRAD *et al.* reported on the mitochondrial DNA from HeLa cells (32). When the normal and cancer mitochondrial DNAs are compared in their contour length, there appear some differences between them. Although both normal liver and cancer mitochondrial DNAs have their main contour length peak around 5 μ, frequency of DNA molecules in multiple length is much higher in cancer mitochondrial DNAs. Moreover, cancer cell mitochondrial DNAs contain a fewer numbers of shorter circular DNA molecules, which are not discernible in normal liver mitochondrial DNA.

The length and shape of DNA molecules were known to be affected by various conditions (43, 44, 49—51), but these variabilities are not great enough to account for dimer-, trimer- or tetramer-sized DNAs. Whether the heterogeneity of cancer mitochondrial DNAs is specific for cancer or not needs further investigation. The majority of the avilable data suggest that normal cell mitochondrial DNAs are homogenous in physical, chemical and morphological properties, but the electron microscopy of mitochondrial DNA on certain organisms including human HeLa-cell (32, 33, 45) demonstrates heterogeneity in mitochondrial DNAs. As for the multiple length mitochondrial DNAs, phage DNAs are shown to have larger S-values when they are at their replicative forms (46, 47). Actually multiple length DNA molecules are observed in ϕx-174 phage (48). In addition, osmotically ruptured mouse L-cell mitochondria also contain multiple length DNA molecules (29). In the present experiment, two dimer-sized DNA molecules appeared in two hundred of circular DNAs from normal liver mitochondria. These facts suggest that heterogeneity of length distributions may related to the rapidity of growth. In view of this, the author investigated the mitochondrial DNA of regenerating rat liver, the data of which will be published elsewhere. There may possibly be other factors

connected with the inhomogeneity of cancer cell mitochondrial DNAs, since cancer cells themselves consist of heterogenous cells and since linear DNA molecules have circular configuration under certain conditions.

In relation to linear molecules of mitochondrial DNA, it is most probable that mitochondrial circular DNAs are broken to pieces, since the majority of linear molecules are of the same length or shorter than the circular molecules. As for the gastric cancer, the nuclear contaminations may induce the linear molecular structure, since gastric cancer mitochondria fraction contains a high percentage of nucleic contamination. On the other hand, a possibility that linear molecules are present *in vivo* in mitochondria cannot be excluded. Further investigations need be carried out on mildly extracted DNA from highly purified mitochondria.

Recently, mitochondrial DNA of mutant *Neurospora* is should to differ from that of a wild type *Neurospora* in density (12), and a morphological defference is shown between wild type yeast mitochondrial DNA and mutant yeast mitochondrial DNA (34). Since Warburg's report on respiration of cancer cell, cancer cell mitochondria have been widely investigated with regard to their functional aspect or phenotypic expression. Further clarification of the relation of mitochondrial function to their genotype will have a paramount significance.

SUMMARY

1. Mitochondria isolated from human liver, hepatoma and gastric cancer contain DNA. The DNA content per mitochondrial protein is about ten times as much in cancer as in normal liver.

2. Human liver, hepatoma and gastric cancer contain circular DNA molecules in their mitochondria. Circular DNAs from normal liver and cancer mitochondria are mostly about 5 μ long, and the frequency of circular DNAs of multiple or shorter length is higher in cancer mitochondrial DNA.

The outline of the present paper was presented at the 26th Congress of Japanese Cancer Association (1967) (52, 53).

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